Role of Facilitative Glucose Transporters in Diffusional Water Permeability Through J774 Cells

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ABSTRACT We have reported previously that in the presence of an osmotic gradient, facilitative glucose transporters (GLUTs) act as a transmembrane pathway for water flow. Here, we find evidence that they also allow water passage in the absence of an osmotic gradient. We applied the linear diffusion technique to measure the diffusional permeability (P_d) of tritiated water (^3H-H_2O) through plasma membranes of J774 murine macrophage-like cells. Untreated cells had a P_d of 30.9 ± 1.8 μm/s; the inhibitors of facilitative glucose transport cytochalasin B (10 μM) and phloretin (20 μM) reduced that value to 15.3 ± 1.8 (50%) and 11.0 ± 0.7 (62%) μm/s, respectively. In contrast, no significant effect on P_d was observed in cells treated with dihydrocytochalasin B (P_d = 28.4 ± 1.5 μm/s). PCMBS (3 mM) inhibited glucose uptake by greater than 95%, and ^3H-H_2O diffusion by ~30% (P_d = 22.9 ± 1.5 μm/s). The combination of cytochalasin B plus pCMBS reduced P_d by about 87% (P_d = 3.9 ± 0.3 μm/s). Moreover, 1 mM pCMBS did not affect the osmotic water permeability in *Xenopus laevis* oocytes expressing the brain/erythtroid form of facilitative glucose transporters (GLUT1). These results indicate for the first time that about half of the total P_d of J774 cells may be accounted for by water passage across GLUTs. Hence, they highlight the multifunctional properties of these transporters serving as conduits for both water and glucose. Our results also suggest for the first time that pCMBS blocks glucose transport without affecting water permeation through GLUTs. Lastly, because pCMBS decreases the P_d of J774 cells, this suggests the presence in their plasma membranes of another protein(s) exhibiting water channel properties.

INTRODUCTION

Water movements across membranes are fundamental in biology (Stein, 1986; Finkelstein, 1987). Because bilayers of lipids extracted from cell membranes exhibit

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much less variation in water permeability than the membranes from which these lipids were obtained, it has been suggested (Stein, 1986; Finkelstein, 1987), that cellular membranes express proteins that facilitate the translocation of water across membranes. Preston and Agre (1991) identified and cloned an integral membrane protein of erythrocytes (CHIP28), and showed that it is a water channel (Preston, Carroll, Guggino, and Agre, 1992) sensitive to mercurials. Other proteins such as facilitative glucose transporters (Fischbarg, Kuang, Hirsch, Lecuona, Rogozinski, Silvestein, and Loike, 1989; Fischbarg, Kuang, Vera, Arant, Silverstein, Loike, and Rosen, 1990; Zhang, Alper, Thorens, and Verkman, 1991), Na^+ glucose cotransporters (Loike, Arant, Kuang, Xu, Cao, Silverstein, and Fischbarg, 1991) and the cystic fibrosis transmembrane conductance regulator (Hasegawa, Skach, Baker, Calayag, Lingappa, and Verkman, 1992) have also been shown to act as conduits for water driven osmotically across membranes.

We examine here water permeation in J774 cells in the absence of an osmotic gradient. We employed the linear diffusion method (Redwood, Rall, and Perl, 1974; Garrick, 1989; Crank, 1957) to measure diffusional water permeability (P_d) across the plasma membranes of a column of packed J774 cells. Inhibitors of facilitative glucose transport significantly reduce P_d in these cells. pCMBS reduces P_d values by about 30% and glucose uptake by greater than 95% in J774 cells, but does not affect osmotic water permeability in *Xenopus laevis* oocytes expressing the brain/erythroid glucose transporter (GLUT1). Our results suggest that GLUTs act as conduits for diffusional water movements, and also suggest the presence of additional protein(s) with water channel properties in the membranes of J77 cells.

MATERIALS AND METHODS

Cell Culture

J774 cells were maintained as suspension cultures at 15×10^5 cells/ml in Dulbecco's modified Eagle's medium containing 5 mM glucose, 10% (vol/vol) heat-inactivated calf serum, and penicillin/streptomycin (DMEM+) as described (Fischbarg et al., 1989). Cell diameters were measured by phase microscopy-split screen micrometry using suspended cells that had been fixed for 10 min in phosphate-buffered saline containing 3.7% paraformaldehyde. The cell average diameter was 21.2 ± 0.4 μm ($n = 100$). Reagents and drugs were from Sigma Chemical Co. (St. Louis, MO); cell culture media and sera were from Gibco Laboratories (Grand Island, NY); radioisotopes were from New England Nuclear (Boston, MA).

Diffusion Studies

The Pd of J774 cells was measured as described (Redwood et al., 1974; Garrick, 1989; Crank, 1957). Cells were concentrated to $20–25 \times 10^6$ cells/ml and resuspended in 0.5 ml of DMEM+. More than 90% of the resuspended cells remained viable as determined by trypan blue exclusion (Loike, Kozler, and Silverstein, 1979).

10 μl of ^{14}C-dextran (0.1 μCi/μl) was added to 0.1 ml of cell suspension for determination of extracellular space. This labeled suspension was drawn into a 6 cm piece of polyethylene tubing (PE205, ClayAdams, Parsippany, NJ). One end of the tube was sealed with soft clay and fused with heat (distally to the clay plug). These sealed tubes were centrifuged at 500 g for 10 min at room temperature to pack the cells further. The polyethylene tubes were then sliced at two sites

(5 mm below the cell-medium interface, and just above the cell-medium interface). The portion of tubing above the cell-medium interface (which contained DMEM+) was frozen with solid CO_2 and was cut into five slices of 1 mm thickness, which were immediately transferred into scintillation vials. The ^{14}C-dextran in the medium in this portion of the tube served to measure extracellular space. The portion of tubing between the cell-medium interface and 5 mm below that interface was discarded because it usually contained imperfectly packed cells. The remaining segment of tubing was then placed into a glass test tube at 37°C. After 5 min, 23 μl (12 μCi/μl) of ^{3}H-H_2O (specific activity = 1.0 mCi/mmol) was added to the open end of the cell column. After 2 min, the excess liquid at the open end was removed by blotting, and diffusion of ^{3}H-H_2O was allowed to proceed. After 30 min, the tube containing the packed cells was frozen with solid CO_2 and sliced from the open end at 1 mm intervals (total: ≈ 10 slices). ^{3}H and ^{14}C radioactivity in these slices was determined in an LKB liquid scintillation counter.

In other experiments, diffusion of ^{3}H-H_2O through the medium was measured as described (Garrick, 1989). To determine the intracellular diffusion coefficient, J774 cells were sedimented in polyethylene tubes as described above, and were lysed by subjecting to three cycles of freezing and thawing.

Coefficients for water diffusion through the column of packed viable cells (D), through extracellular medium (D_1), and through intracellular medium [cells that were frozen and thawed repeatedly; D_2], were determined as described (Garrick, 1989). Numerical values of the diffusional water permeabilities through the packed columns of cells were obtained using the experimental values of D, D_1, D_2, relative extracellular (dextran) space (V_1/V = 0.11 to 0.14; V_1 and V as in Garrick [1989]) and our measured average diameter for J774 cells, and substituting those values into the appropriate equations as described (Crank, 1957; Redwood et al., 1974; Garrick and Redwood, 1977; Garrick, 1989). Deviations are SEM.

Expression of GLUT1 in Oocytes

Xenopus laevis oocytes were prepared as described by Milovanovic, Frindt, Tate, and Windhager, 1991. Oocytes were injected with either 50 nl of water (control) or with 50 ng of GLUT1 cRNA in 50 nl of water. All measurements of osmotic water permeability (P_f) were conducted at room temperature. An oocyte was transferred from culture medium into a glass chamber with Barth's solution (Echevarría, Kuang, Iserovich, Li, Preston, Agre, and Fischbarg, 1993) and left undisturbed for some 30 min. Controls (not shown) indicated that such interval was sufficient for the oocyte volume to reach a steady state. The oocyte was then exposed to a 92% hypotonic challenge. The change in oocyte volume was monitored and its P_f was calculated as described (Fischbarg et al., 1990; Echevarría et al., 1993).

RESULTS

Diffusion and Permeability Coefficients for ^3H–H_2O

The mean values of the diffusion coefficients for ^{3}H-H_2O (D_1, D_2, and D) are given in Table I. The diffusion coefficient for ^{3}H-H_2O through packed cells (D = 3.98 × 10^6 cm^2/s) is considerably lower than the corresponding values for the frozen and thawed cells (i.e., intracellular; D_2 = 12.4 × 10^6 cm^2/s) or cell-free medium (i.e., extracellular; D_1 = 23.8 × 10^6 cm^2/s) media. As expected, an intact plasma membrane generates a marked resistance to water diffusion.

TABLE I

Diffusion Coefficients at 37°C for ^3H-H_2O in J774 Cells

	D_1	D_2	D
		$cm^2/s \times 10^6$	
Control	23.8 ± 0.4 (2)	12.4 ± 1.0 (31)	3.98 ± 0.24 (25)
Phloretin (20 μM)		10.8 ± 1.8 (12)	2.64 ± 0.17 (13)
Cytochalasin B (10.0 μg/ml)		11.6 ± 1.8 (11)	2.96 ± 0.36 (10)
Dihydrocytochalasin B (10 μg/ml)			3.80 ± 0.20 (6)
pCMBS (3 mM)		16.5 ± 1.7 (6)	3.50 ± 0.19 (16)

Numbers of experiments in parenthesis. One experiment represents one tube of packed cells. *D*: water diffusion through the column of packed cells; D_1: through extracellular medium; and D_2: through lysed cells.

Glucose Transport Inhibitors

J774 cells were preincubated for 30 min at 37°C in the presence of cytochalasin B (CytB; 10 μM) or phloretin (Phl; 20 μM). Subsequently, the cells were packed into polyethylene tubes, and the diffusion of ^3H-H_2O was measured at 37°C for up to 30 min. The ^3H-H_2O diffusion coefficient in CytB- or Phl-treated cells was reduced by ~26% and 34%, respectively (Table I). In contrast, dihydrocytochalasin B (diCytB; 20 μM) did not significantly affect the ^3H-H_2O diffusion coefficient in J774 cells (<5% change, Table I). DiCytB was used as a control for CytB, since it also affects the assembly of actin filaments but does not affect facilitative glucose transport (Mannes and Walsh, 1982).

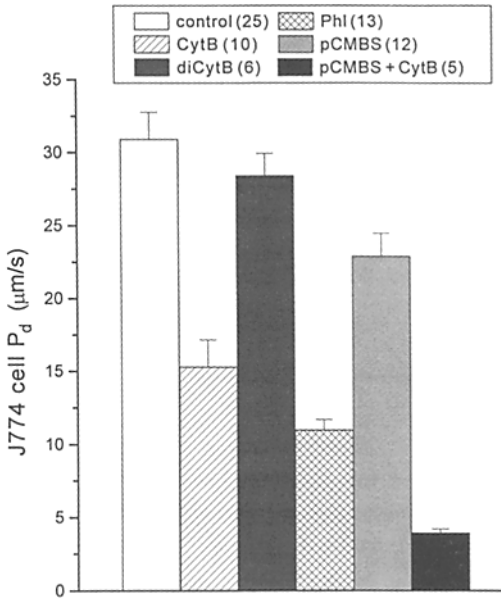

FIGURE 1. The data in Table I have been used to calculate J774 cell membrane diffusional permeabilities. The legend states the experimental conditions and numbers of experiments.

The permeability coefficients P_d were calculated from the diffusion coefficients using the series-parallel pathway model (Redwood et al., 1974). We utilized one value of D per set of packed cells, and average values for D_1 and D_2 from parallel experiments. The P_d value for untreated (control) cells was 30.9 ± 1.8 μm/s. Cells treated with either CytB (10 μM) or Phl (20 μM) exhibited lower P_d values (15.3 ± 1.9 and 11.0 ± 0.7 μm/s, respectively; Fig. 1). These values were significantly different from those in control cells (t test; $P < 0.001$ in both cases). In contrast, J774 cells treated with diCytB (20 μM) exhibited a P_d value of 28.4 ± 1.5 μm/s, which is not significantly different from that in untreated cells (t test; $P \sim 0.3$; Fig. 1).

Effect of pCMBS on Diffusional Water Permeability and Glucose Transport in J774 Cells

The organomercurial pCMBS (3 mM) reduced P_d by 26% in J774 cells (Fig. 1), and inhibited 2-deoxy-D-glucose uptake into these cells in a dose-dependent fashion (Fig. 2). This reduction in P_d is less than that with either CytB or Phl (Fig. 1), but is

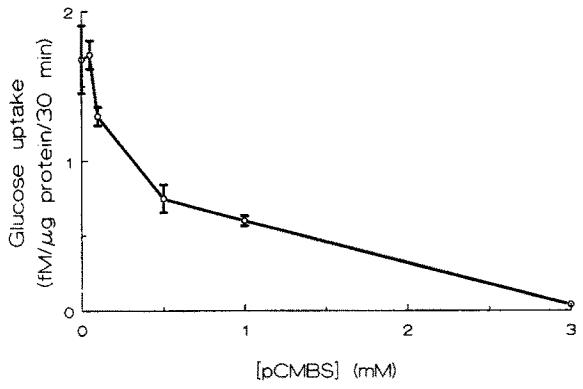

FIGURE 2. Effect of varying concentrations of pCMBS on ^{3}H-2-deoxy-D-glucose uptake. J774 cells maintained on 12-mm coverslips were incubated at 37°C in phosphate buffered saline (Fischbarg et al., 1989) in the presence or absence of varying concentrations of pCMBS. After 30 min, the uptake of labeled deoxyglucose ($n = 3$ coverslips) was measured as described (Fischbarg et al., 1989).

significant (t test, $P < 0.01$). As mentioned above, CytB (10 μM) or pCMBS (3 mM), used singularly, reduced P_d by 50 and 26% respectively (Fig. 1). However, the combination of CytB and pCMBS reduced P_d by 87% (Fig. 1).

Effects of pCMBS on Osmotic Water Permeability in Oocytes Expressing GLUT1

From the results above, pCMBS blocked glucose uptake by >95% but caused only a 30% reduction in P_d across J774 cells. This disparity led us to examine the effects of pCMBS on water permeation through GLUT1 using the oocyte expression system (Vera and Rosen, 1989, 1990; Fischbarg et al., 1990). As previously reported (Fischbarg et al., 1990; Zhang et al., 1991), *Xenopus laevis* oocytes injected with GLUT1 cRNA exhibit a marked increase in osmotic water permeability (P_f) (from 13 to 23 μm/s, Fig. 3 *b*). However, incubation of both control (water injected) and test (GLUT1 cRNA injected) oocytes with 1 mM pCMBS had no effect on P_f (Figs. 3, *a* and *b*), indicating that pCMBS does not block water movements across GLUT1.

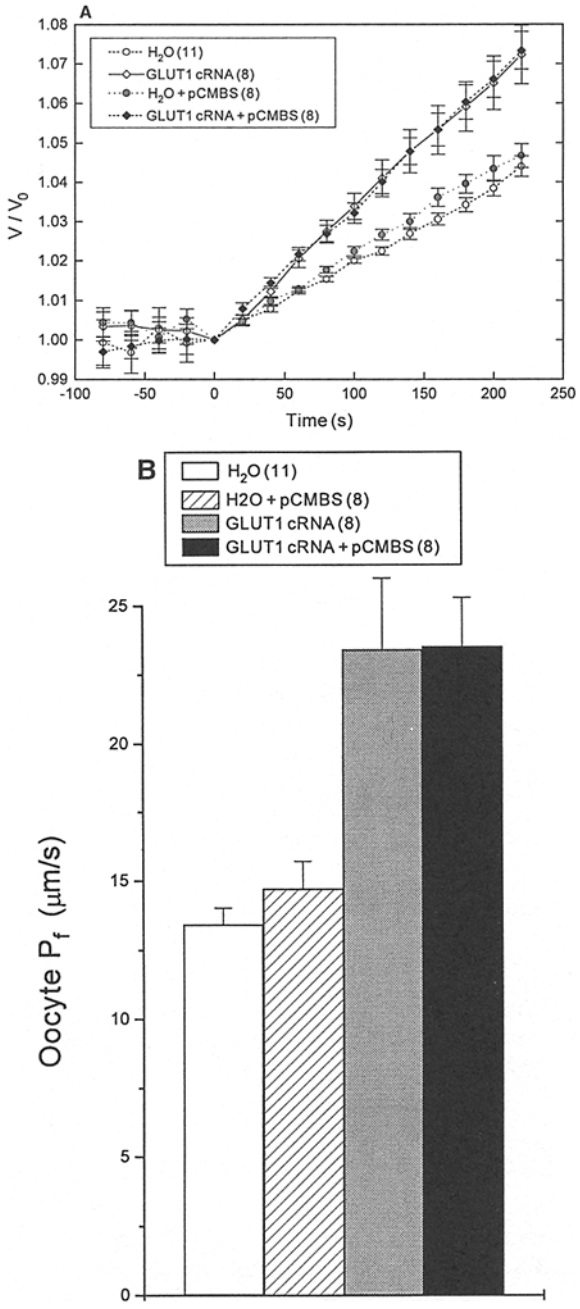

FIGURE 3. (*A*) Effects of pCMBS on *Xenopus* oocytes. All oocytes were injected with either water (*controls*) or GLUT1 cRNA as described under Materials and Methods. For each oocyte, its relative volume represents the ratio of absolute volume over that at zero time. Average relative volumes and their deviations are plotted, and joined by straight lines. Numbers of experiments are given in parenthesis in the panel (each oocyte represents one experiment). (*B*) For each oocyte, a linear fit was found to the segment of the volume vs time curve between $t = 0$ and $t = 220$ s (Fig. 3 *A*), and a P_f value was calculated. Shown: average P_f values with deviations for each experimental group.

DISCUSSION

The diffusional movement of water across J774 cells was significantly retarded by CytB and Phl, and not by diCytB (Table I). CytB and Phl also inhibit glucose uptake by J774 cells in a reversible manner, but the analog diCytB does not affect glucose

uptake (Fischbarg et al., 1989). From the data in Fig. 1, up to 50% of the diffusional permeability to water of the plasma membranes of J774 cells could be mediated by GLUTs.

Xenopus laevis oocytes injected with mRNA encoding GLUT1 exhibit enhanced P_f; this increase in P_f is blocked by Phl (Fischbarg et al., 1990) or CytB (Zhang et al., 1991). These findings led us to propose (Fischbarg et al., 1990; Fischbarg, Kuang, Li, Arant-Hickman, Vera, Silverstein, and Loike, 1993) that GLUTs form channels for transmembrane osmotic water flow. In this light, the most straightforward interpretation of the data presented here is that: (*a*) GLUTs also behave as channels for transmembrane diffusion of water; (*b*) glucose and water traverse the same channel through these transporters.

pCMBS (0.5 mM) inhibits glucose uptake through GLUT1 in both oocytes (Wellner, Monden, and Keller, 1992) and J774 cells (Fig. 2) by 80 and 60%, respectively. Both CytB and pCMBS at the concentrations used here (Fischbarg et al., 1989, and Fig. 2, respectively), inhibit glucose uptake by J774 cells by >95%. Yet, as noted, pCMBS was less effective than either CytB or Phl in inhibiting P_d (Fig. 1). Moreover, pCMBS (1 mM) had no effect on osmotic water permeation in oocytes expressing GLUT1 (Fig. 3, *a* and *b*). This raises the question of why pCMBS reduced P_d in J774 cells by some 26% (Fig. 1). In this connection, Zhang et al. (1991) and Preston et al. (1992) have shown that plasma membrane proteins other than GLUTs serve as water channels. The water conductance of CHIP28 is greatly decreased by mercurial compounds (Preston et al., 1992; Zeidel, Ambudkar, Smith, and Agre, 1992*a*; van Hoek and Verkman, 1992), as is the water conductance of ADH-dependent water channels in urinary epithelia (Ibarra, Ripoche, and Bourguet, 1989). Hence, the additive inhibitory effect of CytB and pCMBS on P_d suggests that pCMBS decreases P_d through effects on water channel proteins other than GLUTs.

Viewed from this perspective, pCMBS binding to sulfhydryl groups on GLUT1 would block glucose transport while sparing the pore through which water traverses GLUTs. In contrast, CytB and Phl block both hexose and water movements across this protein. Hence, whether water passage through a transmembrane protein is affected by sulfhydryl reagents may depend on the location of the sulfhydryl groups relative to the pore.

The Magnitude of the Water Conductance of Facilitative Glucose Transporters

Dempster, van Hoek, de Jong, and van Os (1991), Zeidel, Albalak, Grossman, and Carruthers, 1992*b*), and Echevarría and Verkman (1992) reported that the same inhibitors of glucose transport that we have used did not measurably reduce P_f in membrane vesicles or cultured cells. Nonetheless, our work (Fischbarg et al., 1989, 1990), and that of Zhang et al. (1991), demonstrated that GLUTs have a finite water conductance (Verkman, 1992). The magnitude of this conductance remains at issue.

We have compared the magnitude of water conductance through GLUTs (Fischbarg et al., 1993) to the water conductance through CHIP28 using estimates of the number of copies of GLUT and of CHIP28 expressed by oocytes injected with cRNA encoding each of these proteins (Fischbarg et al., 1990, and Preston et al., 1992). We have calculated that the GLUT water conductance is ~7% of that of CHIP28 (Fischbarg et al., 1993). Therefore, in cells expressing both GLUTs and CHIP28 in

similar amounts (e.g., red blood cells), it will be difficult to detect effects of inhibitors of glucose transport on P_f due to the much larger osmotic conductance of CHIP28. Indeed, it has been reported by several groups that Phl does not measurably affect the osmotic permeability of red blood cells (Macey and Farmer, 1970; Solomon, Chasan, Dix, Lukacovich, Toon, and Verkman, 1983).

Permeability Ratio: Pathways Through Proteins and Lipids

The ratio of P_f (89.6 ± 3.2 μm/s, Fischbarg et al., 1989) to P_d (30.9 ± 1.8 μm/s, Fig. 1) in J774 cells is 2.9 ± 0.1. This is very similar to the 3.1 value obtained by Garrick, Polepka, Cua, and Chinard (1986) for alveolar dog macrophages, suggesting that a substantial fraction of water movement across the plasma membranes of macrophages occurs via transmembrane pores. It has been argued that for most cells (aside from red blood cells and some specialized epithelial cells), their bilayer permeability suffices for the water exchanges necessary for metabolic activity (Finkelstein, 1987). However, in our case, the combination of CytB and pCMBS reduces P_d by 90% (Fig. 1), so 90% of transmembrane water conductance in J774 cells appears to be mediated by membrane proteins. Hence, either macrophages are also specialized cells with high water permeability, or else protein water channels may be more prevalent in cells than has been recognized so far.

A Model for Water Permeation Through GLUTs

We have recently proposed (Fischbarg et al., 1993) a model according to which GLUTs possess a channel with a relatively large diameter (at least 9 Å). Such diameter would confer multifunctional properties to this protein, allowing both water and glucose to traverse it (Fischbarg et al., 1993). In our model, this transporter would possess gates (presumably at the ends of the channel) that fluctuate in position continuously, assuming multiple intermediate conformations between open and closed end states. CytB and Phl could either obstruct the GLUT channel or fix its "gate(s)" in a conformation which would preclude glucose and water movements. In contrast, pCMBS could fix either the gates or the protein channel in a partially open position, so that water (1.4 Å in radius) but not glucose (≈4.0 Å in radius) could traverse the channel. Alternatively, pCMBS may bind to the exofacial domain of GLUT1, thereby restricting access of glucose but not of water to the channel's entrance.

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